PB85-104909

Gas Chromatography/Mass Spectroscopy Analysis of Mutagenic Extracts of Aqueous Chlorinated Humic Acid. A Comparison of the Byproducts to Drinking Water Contaminants

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

1984

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA

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1. REPORT NO. EPA-600/J-84-113	2.
3. PB85-104909	
4. TITLE AND SUBTITLE: Gas Chromatography/Mass Spectroscopy Analysis of Mutagenic Extracts of Aqueous Chlorinated Humic Acid. A Comparison of the Byproducts to Drinking Water	5. REPORT DATE: 1984
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Contaminants (Journal Version): W.E. Coleman, J.W. Munch, W.H. Kaylor, R.P. Streicher, H.P. Ringhand, J.R. Meier	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: U.S. EPA, Health Effects Research Laboratory, CSSB, TMD, 26 W. St. Clair Street, Cincinnati, Ohio 45268	10. PROGRAM ELEMENT NO. ABMC1A
	11. CONTRACT/GRANT NO. In-house
12. SPONSORING AGENCY NAME AND ADDRESS: Health Effects Research Laboratory, ORD, U.S. Environmental Protection Agency, Research Triangle Park, N.C. 27711	13. TYPE OF REPORT AND PERIOD COVERED: Journal Article
	14. SPONSORING AGENCY CODE: EPA 600/11

15. SUPPLEMENTARY NOTES

Environmental Science & Technology, 1984, 18, 674-681.
W. Emile Coleman, Project Officer

16. ABSTRACT

Formation of mutagenic compounds as a result of aqueous chlorination of humics acids has been demonstrated. Solvent extracts responding positively to the Ames test were analyzed by GC/MS in an attempt to identify the mutagenic components. Aliquots of the chlorinated humic solution were also analyzed by closed-loop stripping and GC/MS. Results of GC/MS analyses of methylene chloride and ether extracts and of the closed-loop-stripping extract indicated that trihalomethanes and halogenated acetic acids, acetonitriles, 2-propanones, propenals, propenenitriles, propenes, phenols and thiophenes were formed by the aqueous chlorination of humic acids at neutral pH. The concentrations of eight of the halogenated by-products accounted for about one fourth of the total organic halogen (TOX) content of the aqueous chlorinated humic acid solution. The mass spectra of tentatively identified compounds are included. The similarity of mutagenic compounds identified in this study with compounds previously identified in drinking water suggests that the reaction of chlorine with natural aquatic humics is a likely source of mutagen formation in drinking water. Some data on the production of halogenated compounds from the chlorination of humics in the presence of bromide (Br^-) at various molar concentrations are also included.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT: Release to public	19. SECURITY CLASS (*This Report*): Unclassified	21. NO. OF PAGES: 9
	20. SECURITY CLASS (*This page*): Unclassified	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

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Environ. Sci. Technol. 1984, 18, 674-681

EPA-600/J-34-113
JOURNAL ARTICLE

Gas Chromatography/Mass Spectroscopy Analysis of Mutagenic Extracts of Aqueous Chlorinated Humic Acid. A Comparison of the Byproducts to Drinking Water Contaminants

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■ Formation of mutagenic activity as a result of aqueous chlorination of a model humic acid substrate has been previously demonstrated. In the present study, solvent extracts of solutions of the chlorinated model substrate, which responded positively in the Ames test, were analyzed by gas chromatography/mass spectroscopy (GC/MS) in an attempt to identify the mutagenic components. Results of GC/MS analyses of methylene chloride, ether, and closed-loop-stripping extracts indicated that trihalomethanes and haloacetic acids, -acetonitriles, -propanones, -propenals, -propenenitriles, -propenes, -phenols, and -thiophenes were formed by the aqueous chlorination of humic acid at neutral pH. The concentrations of nine of the halogenated byproducts accounted for about one-fourth of the total organic halogen (TOX) content of the aqueous chlorinated humic acid solution. The similarity of mutagenic compounds identified in this study with compounds previously identified in drinking water suggests that the reaction of chlorine with natural aquatic humic material is a likely source of mutagen formation in drinking water. Some data on the production of halogenated compounds from the chlorination of the model humic acid in the presence of bromide (Br^-) are also included.

Introduction

Byproducts resulting from the reaction of drinking water disinfectants with organic material have been identified as major sources of contamination in drinking water. In 1974 Rook (*1*) demonstrated that chlorination of water containing natural humic substances and inorganic bromide led to formation of chloroform and other chlorobrominated methanes. Later that year Bellar et al. (*2*) reported the formation of trihalogenated methanes following water chlorination, which they suspected was a result of the reaction of chlorine-containing bromine impurities with traces of ethanol. Later studies (*3*, *4*) confirmed that the action of chlorine on humic substances in raw water was the largest source of trihalomethanes in finished drinking water. In 1976, the National Cancer Institute (*5*) published results showing that at high doses chloroform, the most common trihalomethane, could cause cancer in rats.

It is known that halogenated chemicals in addition to the trihalomethanes are formed in the chlorination of drinking water (*6*) and in the chlorination of humic substances (*3*, *7–11*). Generally these chemicals have been more difficult than the trihalomethanes to identify in drinking water, partially because they are more polar and because they are present as a complex mixture of very small quantities of individual chemicals. Furthermore, the bulk of the organic matter in drinking water is relatively nonvolatile, and this has complicated the task of chemical identification.

A number of studies have shown that water chlorination is directly responsible for the formation of mutagenic chemicals in drinking water (*12–14*). More recently Bull et al. (*15*) and Meier et al. (*16*) found that chlorination of a commerically available humic acid (Fluka) and aquatic humic materials separated from well-characterized lakes (Black Lake and Lake Drummond) resulted in the formation of mutagenic activity similar to that produced by mutagenic constituents of drinking water. They reported that the specific mutagenic activity produced by the chlorination of Fluka humic acids at a Cl:C molar ratio of 1.0 was comparable to activity induced in humic and fulvic acids derived from aquatic sources. On the basis of these latter findings as well as economy, ready availability, temporal variability of natural water and humic materials, and the need for controlled and reproducible experimental procedures, the potential health effects of chlorination byproducts and other contaminants is currently being assessed by examining the biological consequences of chlorination on this selected model substrate. Even though the Fluka humic material was derived from a soil source, the chlorination byproducts presented in this paper are similar to many organic contaminants that we have detected in drinking waters.

This paper describes chemical techniques such as derivatization, solvent extraction, and closed-loop stripping and results of analyses achieved by computer-assisted capillary column gas chromatography/mass spectroscopy (GC/MS). These techniques were employed in an attempt to isolate, identify, and quantify mutagenic compounds in methylene chloride and ether extracts of the model chlorinated humic acids that responded positively in the *Salmonella*/microsomal mutagenicity assay described by Ames et al. (*17*). Many of the compounds are tentatively identified because of the unavailability of authentic standards.

Experimental Section

Preparation of Chlorinated Humic Material. (1) Fluka Humic Acid. Humic acid solutions were prepared according to Meier et al. (*16*) with a commercial humic material (Fluka, Switzerland) and distilled water. The pH 7 soluble portion of Fluka humic material which was used in this study was found to contain 92% humic acid and 8% fulvic acid as determined by using the classical precipitation procedure described by Christman et al. (18). The results of elemental analyses of the Fluka humic material were the following: C, 51.5%; H, 4.6%; O, 29.3%; N, 0.7%; S, 3.5%; ash, 8.4%. The chlorine water that was used for chlorination of the humic acid solutions was prepared by bubbling chlorine gas into a NaOH solution (*16*).

The chlorination of humic material was carried out immediately after the preparation of the chlorine water. Once the TOC of the humic solution was determined, enough of the concentrated humic solution was added to a 250-mL amber bottle so that when diluted to 200 mL, the TOC concentration would be 1.0 g/L. A ratio of 1 equiv of chlorine (as Cl) per mol of carbon (as TOC) was chosen for laboratory experiments. More than 99% of the chlorine equivalents were consumed after 90 h by using this Cl:C ratio. At the end of the reaction time pH was measured, was typically 2.5–3.0 in samples initially chlorinated at pH

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7, and was typically 7.0–7.5 in samples initially chlorinated at pH 11. The samples were stored at 4 °C until time for analysis.

It should be noted that the 1 g/L TOC of the humic acid and the 1:1 Cl:C molar ratio used in this study are based on previous studies that examined the reaction conditions under which mutagenicity is produced. The effect of TOC concentration on mutagenicity levels and the reasons for selecting 1 g/L TOC in the model humic acid substrate studies have been previously discussed by Meier et al. (*16*). Moreover, absolute concentration should not be a factor in determining products. Rather, the chlorine to carbon ratio is more significant, and it is similar to the ratio used in drinking water treatment.

(2) Ohio River Humic Material. Humic material isolated from the Ohio River was chlorinated by using the previously described procedure at pH 7. The TOC was 1.0 g/L, and chlorine was added at a 1:1 Cl:C molar ratio. The humic material (fulvic plus humic acid fractions) was isolated from river water by adsorption on XAD-8 resin, followed by ion-exchange chromatography using the method described tby Thurman and Malcolm (*19*). No attempt was made to separate the fulvic and humic acid fractions for purposes of this study. However, separation of the acids on another portion of the sample indicated that the ratio of fulvic acid:humic acid was approximately 9:1.

(3) Chlorination of Fluka Humic Acid in the Presence of Bromide. To study the effect of bromide on the reaction of chlorine with humic material, a series of Fluka humic acid solutions (all 1 g/L TOC) were initially chlorinated at pH 7 after varying amounts of potassium bromide (KBr) had been added to the humic solution. The concentration of Kbr ranged from 0 to 2.0 g/200 mL. The molar ratio of Cl:C was 1:1, and the molar ratios of Br^- were 0, 0.01, 0.05, 0.1, 0.5, and 1. Following the addition of chlorine, the samples were allowed to stand 90 h at room temperature to ensure complete reaction. The acidic solutions, pH ~2.6, were then stored at 4 °C until ready for analysis.

Extraction Procedures. (1) Methylene Chloride. A 10-mL aliquot of chlorinated humic solution was pipetted into a 40-mL glass vial having a Teflon-backed septum screw cap. It was diluted with 10 mL of low organic reagent water (TOC < 0.15 ppm) to help reduce foaming during extraction. Samples chlorinated at pH 11.5 were acidified to pH 2 just before extraction. Fifty-two micrograms each of 1-chlorohexane, 1-chlorooctane, and 1-bromo-4-fluorobenzene in acetone was added as internal standards to the vial to monitor extraction recoveries and GC/MS instrumentation performance. Finally, 2 mL of Burdick & Jackson "distilled in glass" methylene chloride was added. The vial was capped and mechanically shaken for 10 min. A minimum settling time of 15 min was allowed before removing a 2-μL aliquot of the methylene chloride for capillary column GC/MS analysis. Portions of the aqueous and solvent phases were also used for mutagenesis testing. Before assaying, a solvent exchange with dimethyl sulfoxide (Me_2SO) was performed with N_2 to remove the methylene chloride. This step was necessary because of the toxicity of methylene chloride to the test bacteria.

(2) Diethyl Ether. Ether extraction was performed to enable the identification and quantification of di- and trichloroacetic acids as their methyl esters. Twenty milliliters of chlorinated humic solution was diluted with 20 mL of reagent water and extracted twice with 20-mL portions of ether. The extracts were combined, and a 5-mL aliquot was removed and concentrated at 40 °C by nitrogen evaporation to 500 μL. Part of this concentrated ether extract was then methylated according to the method of Humbert et al. (20) with 3-methyl-1-*p*-tolyltriazene and allowed to react at room temperature for 2 h. After methylation, the ether extract was analyzed by capillary column GC/MS. The remaining unmethylated ether extract as well as a portion of the aqueous phase was tested for mutagenicity. A solvent-exchange step, as described previously, was performed on the ether extract before mutagenicity testing.

(3) Acetylation/Methylene Chloride Extraction. A modified version of the acetylation procedure established by Coutts et al. (*21*) was used in this study. Coutts et al. added 10 g (excess) of $NaHCO_3$ buffer to 250 mL of aqueous solution containing phenols just before the addition of 500 μL of acetic anhydride. After the acetic anhydride was added, the solution was shaken until the evolution of CO_2 had ceased. To avoid any losses of the newly formed esters due to the purging action of the evolving CO_2, we chose Na_2HPO_4 as an alternative buffer. Its pK_b is similar to that of $NaHCO_3$ but evolves no gases upon reaction with an acid.

Two hundred fifty milliliters of chlorinated humic acid were acetylated and then extracted with two 10-mL aliquots of methylene chloride. The extracts were not pure methylene chloride solution but instead contained a substantial layer of emulsion. The combined extract was allowed to stand for 30 min, during which the emulsion layer narrowed and a small aqueous layer formed above it. The homogeneous methylene chloride solution below was removed with a syringe. The remaining aqueous phase/emulsion mixture was then extracted with 10 mL of methylene chloride and allowed to stand for 10 min, and again the methylene chloride phase was removed with a syringe and combined with the two other extracts. The combined volume of methylene chloride retrieved was about 27 mL.

The second modification involved the means of concentration. Coutts et al. (*21*) used a gentle stream of nitrogen to concentrate the whole extract down to 20 μL. We used a micro-Snyder distilling column to concentrate the extract down to 1 mL, thus reducing losses of solutes compared with nitrogen evaporation. The 1-mL solution was further concentrated to 10 μL via nitrogen evaporation at 40 °C.

Closed-Loop-Stripping (CLS) Analysis. Closed-loop stripping was developed in Switzerland by Grob (*22*) in 1973 for analysis of clean water sources and was later validated by Grob and Zürcher (*23*) in 1976. A complete historical background of CLS, its operation, and its applications to drinking water health effects research have been discussed by Coleman et al. (*24*). Modification of the CLS system and typical analytical conditions used in this study were discussed in a recent paper by Coleman et al. (*25*).

Closed-loop-stripping analysis was performed in this study to provide qualitative information on compounds in the aqueous chlorinated model humic acid solution that were present below the detection limits of the solvent extraction methods.

Computer-Assisted Fused Silica Capillary Column GC/MS System. GC/MS analyses and quantification results were preformed on a Finnigan Model 3300 quadrupole mass spectrometer equipped with an Incos Model 2300 data system computer. A Carlo-Erba Fractovap 4160 gas chromatograph was equipped with a Grob-designed splitless injector and a 60 m long × 0.25 mm i.d. wall-coated-open-tubular fused silica SE-30 column. The GC

and GC/MS analytical conditions have been reported previously by Coleman et al. (25). The GC column and conditions used for the analysis of samples from the chlorination of humic acid in the presence of bromide were similar, except that the liquid phase was DB1 and the helium head pressure was 1.35 kg/cm^2 (19 psi).

Quantification. A standard addition technique, which takes into account the effect of sample matrices, was used for quantification of identified compounds. Chlorinated humic material was initially extracted by the procedure outlined earlier to establish the level of the compounds of interest. After the solvent extract was removed, the aqueous material remaining was split into two portions. One portion was spiked with internal standards, and the other portion was spiked with internal standards and known concentrations of the compounds to be quantified (1.5–40 μg/mL). Both samples were solvent extracted again by the usual procedures and the extracts analyzed by GC/MS. If any of the compounds of interest were identified in the extract of the unspiked samples, those GC peak areas were subtracted from the GC peak areas of those compounds in the spiked sample extract, and percent recovery of calculations (26) were made by using eq 1. T

$$\% \text{ recovery} = 100(Y - X)/T \qquad (1)$$

is the spike or standard level, Y is the measured concentration of a spiked aliquot, and X is an estimate of the background concentration based on the measurements of three unspiked aliquots. All peak areas were adjusted by using an internal standard before making recovery calculations. Quantification of individual compounds, incorporating recovery efficiency and response factors, was performed by using the procedure reported by Coleman et al. (25).

In general, peak areas from the reconstructed ion chromatogram (RIC) were used for amount determinations. In some cases of trace levels or interfering GC peaks, single ion quantification (25) was performed using the area of a single characteristic ion to calculate both response factor and amount. Where authentic standards were not available, estimated concentrations based on the recovery and response of an internal standard were calculated by using peak areas from the RIC.

Mutagenicity Assays. The *Salmonella* histidine reverse mutation assay described by Ames et al. (17) was used for assessment of mutagenicity. The assays were performed with *S. typhimurium his*$^-$ strains TA 98 and TA 100. Rat liver homogenate fraction (S9) was omitted from the assays because the mutagenic activity present in the model-chlorinated humic acid was previously found to not require metabolic activation (16).

Synthesis of 1,1,1-Trichloro-2-propanone. 1,1,1-Trichloro-2-propanone was prepared from 1,1-dichloro-2-propanone (27).

Synthesis of 3,3-Dichloropropenal. The preparation of 3,3-dichloropropenal was carried out by using a modification of the method of Kharasch et al. (28). Bromotrichloromethane is added to vinyl acetate in the presence of a radical initiator, followed by elimination of acetyl bromide and HCl. In our procedure, benzoyl peroxide was used as the initiator instead of acetyl peroxide. Also, a 4:1 CCl_3Br:vinyl acetate molar mixture was heated until the reaction was initiated (~55 °C), followed by dropwise addition of vinyl acetate until a molar equivalent had been added. Kharasch et al. simply heated a 2:1 CCl_3Br:vinyl acetate molar mixture to 55 °C for 3 h.

Blanks. Procedural blanks were incorporated into the analytical scheme. Solvent blanks were analyzed, as well as methylated and unmethylated extracts of reagent water and solutions of unchlorinated humic material.

Table I. Efficiency of Extraction of Mutagenic Activity from Chlorinated Fluka Humic Acid with Methylene Chloride or Diethyl Ether

fraction	TA 98 mutagenicity: net revertants/mL[a] ± SE	TA 98 mutagenicity: % recovered	TA 100 mutagenicity: net revertants/mL[a] ± SE	TA 100 mutagenicity: % recovered
methylene chloride extraction				
before extraction	443 ± 24	(100)	2415 ± 86	(100)
after extraction				
aqueous phase	212 ± 22	48	932 ± 134	39
CH_2Cl_2 phase	201 ± 18	45	1006 ± 73	42
diethyl ether extraction				
before extraction	299 ± 34	(100)	2470 ± 123	(100)
after extraction				
aqueous phase	ND[b]		ND[b]	
ether phase	255 ± 28	85	2608 ± 60	106

[a] Values represent specific mutagenic activities based on volumes equivalent to the original amounts before extraction. Values were calculated as the initial slopes ± standard error of the slopes from the dose–response curves by using least-squares regression analysis of the data. Dose–response assays were conducted at four dose levels using duplicate plates per dose. [b] ND = not detected (i.e., response at any dose level was less than 2-fold above background).

Results and Discussion

Since Bull et al. (15) and Meier et al. (16) demonstrated that aqueous solutions of model chlorinated humic acid exhibited mutagenic activity in the *Salmonella* histidine reversion assay (Ames test), chemical approaches were taken to isolate and identify biologically active compounds. Solvent extractions of chlorinated humic acid solutions using separate extractions of pentane, methylene chloride, and diethyl ether were attempted in efforts to isolate the mutagenic chemicals. In general, GC/MS analyses of the solvent extracts indicated that a similar profile of compounds were detected in all extracts, but the relative level of compounds detected as GC peaks was greatest in the methylene chloride extract, followed by diethyl ether and then the pentane extract. In contrast, when the methylene chloride and ether extracts were assayed for mutagenic activity in the Ames test, the results (Table I) showed that ether extraction removed nearly all of the mutagenic activity (both for TA 98 and TA 100) from the original solution while the methylene chloride extraction removed less than half of the mutagenicity. This suggests that the ether extracts probably contained highly polar compounds that were not suitable to gas chromatography such as acids and phenols and that these polar compounds could be responsible for the increased mutagenic activity. The ether extracts were subsequently methylated (20) to form esters of the acids. Di- and trichloroacetic acids were the only two acids detected when the referenced methylation procedure (20) was used.

Results of GC/MS analyses of methylene chloride and ether extracts of solutions of chlorinated humic material are shown in Table II. The compounds listed in Table II were the major components detected by direct extraction with methylene chloride or ether. All compounds were quantified by analysis of the methylene chloride extracts except di- and trichloroacetic acids which were quantified from the ether extracts after methylation. The concentrations of the compounds given in Table II account for approximately one-fourth of the total organic halogen (TOX) content of the model-chlorinated humic acid solution. The solvent extracts were not concentrated further,

Table II. Compounds Identified and Quantified in Solvent Extracts of Chlorinated Humic Material

humic material source	Ohio River	Fluka	Fluka
prechlorination pH	7	7	11.5
postchlorination pH	3.1	2.2	7.1
extraction pH	3.1	2.2	2.2

	concn, μg/mL of humic material			
compd	Ohio River	Fluka	Fluka	mutagenicity (ref)
chloroform[a,d]	65.7	22.7	77.7	– (*29*)
dichloroacetonitrile[a,d]	0.2	3.5	4.2	+ (*29*)
trichloroacetonitrile[a,d]	<0.1[e]	0.8	<0.1	– (present study)
1,1-dichloro-2-propanone[a,d]	0.6	2.0	<0.1	+ (present study)
1,1,1-trichloro-2-propanone[a,d]	11.1	8.6	2.1	+ (present study)
1,1,1,3-tetrachloro-2-propanone[b]	NF[f]	<0.1	NF[f]	?
1,1,3,3-tetrachloro-2-propanone[c]	NF[f]	2.6	NF[f]	+ (*30*)
pentachloro-2-propanone[c]	1.1	5.5	NF[f]	+ (*30*)
3,3-dichloro-2-butanone[b,d]	0.4	NF[f]	NF[f]	?
dichloroacetic acid[a,c,d]	14.0	28.0	34.0	– (*31*)
trichloroacetic acid[a,c,d]	35.0	49.0	88.0	– (*31*)
TOX	350	350	540	
% TOX[g]	28	23	28	

[a]Quantification based on recovery and response of authentic standards. [b]Quantification is estimated on the basis of recovery and response of an internal standard, 1-chlorohexane; authentic standards not available. [c]Quantitated as the methyl ester. [d]Previously identified in drinking water. [e]< = present, but less than the quantification limit of 0.1 μg/mL. [f]NF = not found; may or may not be present at <0.1 μg/mL. [g]Percent TOX accounted for, on the basis of quantification of the above compounds with authentic standards.

as a portion of each extract was shared for the mutagenicity assays. The quantification limit would be lower and probably more compounds would have been detected if the solvent volumes were reduced by nitrogen evaporation, but it was felt that concentration of the sample in this manner might cause loss of some volatile mutagens. We later demonstrated that significant losses of chloroform, dichloroacetonitrile, trichloroacetonitrile, 1,1-dichloro-2-propanone, and 1,1,1-trichloro-2-propanone occurred when the extracts were concentrated 10-fold. Many investigators have shown that chloroform levels resulting from the chlorination of drinking water and humic material increase with increasing pH as was indicated in Table II with the Fluka humic acid chlorinated at pH 11.5. Compounds observed in extracts of chlorinated Fluka humic acid and Ohio River humic material (Table II) are similar to compounds that have been detected in Cincinnati drinking water throughout this laboratory's experience of investigating methodologies for measuring trace organics. The Ohio River is the source of drinking water for the city of Cincinnati, Oh. The significant difference between the di- and trichloroacetonitrile concentrations in the chlorinated Fluka humic acid and Ohio River humic material could result from a difference in the nitrogen content of the two humic samples, which was undetermined in the Ohio River humic sample but was known to be 0.7% in the Fluka humic acids. The intent of Table II is not to directly compare results of analyses of the Fluka humic acid with

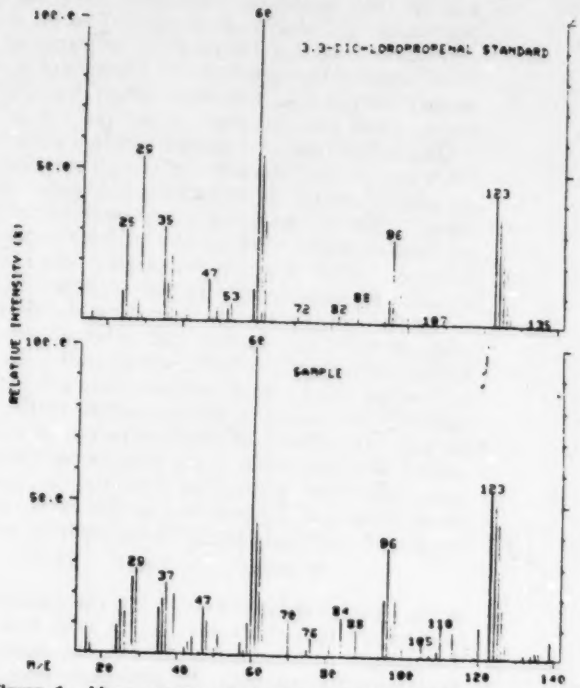

Figure 1. Mass spectra of standard 3,3-dichloropropenal and dichloropropenal identified in the CLS extract of chlorinated Fluka humic acids. RRT of both were identical, 0.776 relative to 1-bromo-4-fluorobenzene.

Ohio River humic material but to show that similar chlorination byproducts are produced in water even though the fulvic acid:humic acid ratios are quite different.

Several of the compounds identified and quantified in the chlorinated humic acid samples and listed in Table II are known to be mutagens (*29–31*; present study). On the basis of the mutagenic activities of these compounds on a per milligram basis and the levels at which they are present, it does not appear that these compounds can account for a significant proportion of the observed mutagenic activity present in the model-chlorinated humic acid samples. It is possible that some of the remaining constituents (Table III) for which standards were not available (thus, are not quantified) may account for a significant portion of the mutagenic activity. For example, chloroacroleins (i.e., chloropropenals) as a class have been found to be highly potent mutagens in the range of 100–700 revertants/nmol (*32*). Rosen et al. (*32*) tested 2,3-dichloropropenal in the Ames test and found it to be a potent mutagen. Then possibly the presence of very small amounts of dichloropropenal, which was identified in the CLS–GC/MS analysis and recently confirmed as 3,3-dichloropropenal (Figure 1), would make a significant contribution to the mutagenicity of the chlorinated humic acid samples. The concentration of 3,3-dichloropropenal in the Fluka humic acid solution chlorinated at neutral pH was 60 μg/L. 1,1,2,3,3-Pentachloropropene, which was also detected in the CLS extract, was reported by Nestmann et al. (*33*) as one of the mutagenic constituents identified in pulp and paper mill effluents.

A closed-loop-stripping analysis was performed on a 1-L sample containing 8 mL of chlorinated Fluka humic acid solution and reagent water for qualitative comparison to the methylene chloride extracts. Some of the compounds detected in the CLS extracts are listed in Table III and have been tentatively identified by GC/MS, using manual interpretation of many of the mass spectra, since most of

Table III. GC/MS Identification of Some Byproducts Resulting from the Chlorination of a Fluka Humic Acid

GC peak[a]	compd	method of isolation[b]	EI mass spectra, m/z (rel abundance %)
1	chloroform[c]	ME, CS	
2	trichloroacetonitrile[c,d]	ME, CS	108 (100), 110 (70), 73 (26), 47 (25), 35 (21), 112 (15), 82 (14), 38 (13) M⁺: 143 (0)
3	bromodichloromethane[c]	CS	
4	dichloroacetonitrile[c,d]	ME, CS	74 (100), 82 (64), 84 (46), 76 (35), 47 (19), 35 (15), 38 (11), 49 (7) M⁺: 109 (0)
5	1,1-dichloro-2-propanone[c,d]	ME, CS	43 (100), 15 (23), 27 (15), 63 (10), 83 (8), 45 (7), 85 (6), 47 (6) M⁺: 126 (0)
6	3,3-dichloro-2-butanone[c,d]	CS	43 (100), 77 (22), 105 (19), 97 (18), 61 (13), 99 (11), 41 (10), 15 (9) M⁺: 140 (1)
7	dibromochloromethane[c]	CS	
8	3,3-dichloropropenal[d]	CS, EE	60 (100), 61 (55), 29 (54), 123 (42), 124 (36), 62 (34), 35 (31), 96 (27) M⁺: 124 (36)
9	dichloropropenenitrile[c]	CS	86 (100), 60 (91), 121 (89), 51 (85), 123 (67), 62 (50), 88 (46), 85 (38) M⁺: 121 (89)
10	1,1-dichloro-2-butanone[c,d]	CS	57 (100), 29 (52), 27 (40), 41 (25), 26 (16), 83 (16), 48 (14), 39 (13) M⁺: 140 (0)
11	bromochloroacetonitrile[c,d]	CS	74 (100), 76 (33), 81 (12), 79 (11), 155 (9), 47 (9), 118 (7), 153 (7) M⁺: 153 (7)
12	1,1,1-trichloro-2-propanone[c,d]	ME, CS	43 (100), 15 (15), 125 (9), 47 (8), 97 (7), 127 (6), 82 (6), 83 (6) M⁺: 160 (0)
13	2,2-dichloro-3-pentanone[c,d]	CS	57 (100), 29 (30), 97 (17), 27 (17), 55 (17), 99 (11), 61 (11), 91 (9) M⁺: 154 (0)
14	trichloropropenenitrile[c,d]	CS	94 (100), 85 (97), 155 (82), 157 (78), 96 (74), 120 (72), 122 (61), 47 (49) M⁺: 155 (82)
15	bromoform[c]	CS	
16	1,1,1-trichloro-2-butanone[c,d]	CS	57 (100), 29 (39), 82 (38), 139 (31), 84 (20), 141 (20), 75 (20), 117 (18) M⁺: 174 (0)
17	hexachloroethane[c]	CS	
18	pentachloropropene[c,d]	CS	179 (100), 177 (73), 181 (51), 83 (51), 107 (40), 72 (39), 85 (35), 37 (26) M⁺: 212 (8)
19	1,1,1,3,3-pentachloropropanone[d]	ME	83 (100), 117 (72), 119 (70), 85 (66), 47 (35), 111 (26), 121 (26), 82 (26) M⁺: 228 (1)
	tetrachlorothiophene[c]	CS	222 (100), 143 (89), 79 (88), 115 (88), 71 (87), 220 (86), 141 (81), 185 (78) M⁺: 220 (86)
	bromotrichlorothiophene[c,f]	AC/ME	185 (100), 187 (98), 71 (75), 115 (74), 44 (58), 266 (56), 80 (55), 144 (54) M⁺: 264 (22)
	tetrabromothiophene[c,d]	AC/ME	400 (100), 80 (81), 402 (73), 398 (66), 161 (49), 79 (41), 159 (39), 81 (33) M⁺: 396 (20)
	tetrachlorocyclopropene[c]	CS	141 (100), 143 (99), 71 (77), 106 (69), 108 (50), 36 (43), 145 (38), 35 (32) M⁺: 176 (23)
	pentachloro-3-buten-2-one[c]	CS	123 (100), 125 (65), 60 (20), 82 (17), 95 (17), 84 (16), 127 (15), 97 (14) M⁺: 240 (0)
	hexachlorocyclopentadiene[c,d]	CS	237 (100), 235 (62), 239 (57), 119 (46), 130 (39), 95 (39), 141 (35), 143 (33) M⁺: 270 (5)
	bromodichlorophenol, acetate ester[c,f]	AC/ME	43 (100), 97 (43), 15 (38), 242 (35), 240 (34), 244 (25), 62 (18), 99 (9) M⁺: 282 (0)
	tribromophenol, acetate ester[c,d]	AC/ME	43 (100), 15 (43), 330 (42), 332 (37), 62 (36), 61 (23), 141 (23), 143 (20) M⁺: 370 (0.2)
	dichlorodihydroxybenzene, diacetate ester[c,f]	AC/ME	43 (100), 178 (44), 180 (31), 15 (19), 85 (11), 220 (11), 113 (8), 50 (8) M⁺: 262 (0)

Table III (Continued)

GC peak[a]	compd	method of isolation[b]	EI mass spectra, m/z (rel abundance %)
	dibromodihydroxybenzene, diacetate ester[e,j]	AC/ME	43 (100), 15 (21), 268 (17), 50 (12), 266 (10), 270 (9), 129 (6), 310 (5) M⁺: 350 (0)
	trichlorodihydroxybenzene, diacetate ester[e,j]	AC/ME	43 (100), 212 (33), 214 (32), 15 (26), 216 (12), 84 (7), 119 (6), 121 (6) M⁺: 296 (0)

[a] Refers to numbered GC peaks in Figure 2. [b] ME = CH_2Cl_2 extraction; EE = ether extraction; AC/ME = acetylation/CH_2Cl_2 extraction; CS = closed-loop stripping. [c] Compounds that have been identified in finished drinking water. [d] These compounds have been confirmed by GC/MS with authentic standards; their mass spectra may not be in the mass spectral data bases. [e] Structures based on manual interpretation of mass spectra; authentic standards not available. [f] Detected in samples having a Br:Cl:C molar ratio of 0.01:1:1. [g] Detected in samples having a Br:Cl:C molar ratio of 0.1:1:1.

the standards are presently unavailable.

Other investigators have reported similar chlorination byproducts in their studies. Giger et al. (*34*) chlorinated water from Lake Zürich (pH 8; total organic carbon 2 mg/L; residual chlorine 10 mg/L) and reported the formation of several α-chloroketones: 3,3-dichloro-2-butanone 2,2-dichloro-3-pentanone, 1,1,1-trichloro-2-propanone, and a dichloro-3-hexanone isomer. They suggested that larger molecules were the precursors which yielded these ketones through cleavage reactions.

Oliver (*8*) recently reported the presence of dihaloacetonitriles (DHAN) in 10 drinking water samples from diverse locations in southern Ontario, Canada. He reported a range of concentrations from 0.3 to 8.1 ppb and estimated that the average molar DHAN was about 10% of the average molar trihalomethane concentration. Oliver (*8*) also conducted aqueous chlorination experiments on aquatic fulvic acids and two species of aquatic algae. He showed that these materials also yielded DHANs under conditions similar to those used for water treatment. The levels of DHANs that Oliver found in drinking water agree favorably with our results since the levels we saw were as high as 4.2 ppm and the TOC of the humic acid in the present study was approximately 500–1000 times the TOC measured in drinking water sources, which may range from 2 to 20 mg/L TOC.

Trehy and Bieber (*35*) also found that chlorination of natural water in southern Florida was responsible for the formation of DHANs. They showed experimentally that DHANs were formed also from the aqueous chlorination of several amino acids.

Pereira (*36*) studied the reaction of aqueous hypochlorous acid with α-amino acids and dipeptides and showed that HOCl by oxidative decarboxylation converted several α-amino acids into a mixture of corresponding nitriles (major) and aldehydes (minor products). Humic substances with amino acid moieties appended to the ring system could be a source for halonitrile formation. The humic acid structures proposed by Kleinhempel (*37*) show such a moiety. Morris (*38*) reported that hypochlorous acid is 10^4 times more reactive toward nitrogenous compounds than hypochlorite ion. This fact may help explain the presence of haloacetonitriles at low pH in our studies.

Johnson et al. (*9*) reported a large number of chlorinated organic compounds that formed as a result of aqueous chlorination of aquatic humic materials. The products identified in their studies were chlorinated aliphatic straight-chain acids dominated by di- and trichloroacetic acid and the chlorinated dicarboxylic acids: succinic, fumaric, and maleic acids. These researchers did not report any chlorinated aromatic products in their studies.

Quimby et al. (*7*) identified similar chlorinated aliphatic acids; however, they also reported the tentative identification, based on GC retention time, of several halogenated phenols and aromatic carboxylic acids. The Br:Cl:C molar ratios used by Quimby et al. were 0.1:0.4:1. Rook (*3*) also reported chlorinated aromatic compounds in heavily chlorinated natural waters (Cl:C molar ratio ~3:1).

Because of this difference in reported results concerning the presence of chlorinated aromatic compounds, and the fact that the ether extracts in our studies exhibited more mutagenic activity than the methylene chloride extracts, we were prompted to investigate this problem. When aqueous humic material is subjected to chlorination, the resulting reactions include oxidations, electrophilic substitutions, and electrophilic additions. The oxidized byproducts of chlorination are frequently polar compounds which neither extract well from the aqueous phase nor chromatograph well once extracted. Phenols and other alcohols are common examples of such compounds. Both of these isolation problems can be overcome by esterification of these alcohols. The acetylation method adapted from Coutts et al. (*21*) was employed to analyze phenolic compounds in the chlorinated humic acid solutions. We have tentatively identified by GC/MS several halogenated phenolic compounds that were analyzed in model-chlorinated humic acid samples having a Cl:C molar ratio of 1:1 and Br:C molar ratios ranging from 0:1 to 1:1. The following compounds were detected in the sample having a Br:Cl:C molar ratio of 0.01:1:1: 4-chlorophenol (<0.1 μg/mL), 2,4-dichlorophenol (<0.1 μg/mL), 2,4,6-trichlorophenol (0.1 μg/ml), bromodichlorophenol (two isomers), dichlorodihydroxybenzene (two isomers), and trichlorodihydroxybenzene (see Table III). Only the chlorinated species were detected in the absence of bromide.

McCreary and Snoeyink (*39*), in their work with the reaction of free chlorine and humic substances before and after adsorption on granular activated carbon (GAC), reported the presence of chlorophenols in the chlorinated influent and chlorinated dihydroxybenzenes in the carbon extract. They expressed uncertainty as to whether the latter compounds were formed on GAC or were simply too dilute in the influent to be measured without prior concentration by GAC.

The effect of pH on the products of chlorination is substantial. Previous work (*16*) has shown that mutagenicity is substantially reduced upon chlorination of humic acid at high pH. In the present study, samples in which the pH was allowed to drop to ~2.5 after initial chlorination at neutral pH showed chlorinated propanones and acetonitriles as major constitutents. However, in an experiment where the pH did not drop below 9.3 after initial chlorination at pH 11, these compounds were not found (data not shown). This reduction is consistent with the decline in the ability of chlorine to undergo electrophilic substitution reactions due to the unfavorable

Table IV. Effect of Bromide (Br^-) on the Production of Haloacetonitriles (XACNs), Halo-2-propanones (XPROPs), and Trihalomethanes (THMs) during Chlorination of Fluka Humic Acid at Neutral pH

molar ratio Br:Cl:C	ΣXACNs/ΣTHMs × 100[a]	ΣXPROPs/ΣTHMs × 100[a]	predominating THM	predominating XACN	predominating XPROP
1:1:1	0.5	1.3	bromoform	dibromoacetonitrile	1,1,1-tribromo-2-propanone
0.5:1:1	<0.1	0.9	bromoform	dibromoacetonitrile	1,1,1-tribromo-2-propanone
0.1:1:1	0.6	5.7	bromoform	bromochloroacetonitrile	1,1-dibromo-1-chloro-2-propanone
0.05:1:1	1.7	6.0	dibromochloromethane	dichloroacetonitrile	1,1,1-trichloro-2-propanone
0.01:1:1	3.6	8.3	chloroform	dichloroacetonitrile	1,1,1-trichloro-2-propanone
0:1:1	11.0	53.0	chloroform	dichloroacetonitrile	1,1,1-trichloro-2-propanone

[a] The percentages in columns 2 and 3 were determined by summing the reconstructed ion current (RIC) areas of the individual HACNs and XPROPs and comparing them to the summation of the RIC areas of the THMs. pH of all solutions was about 2.5

$HOCl:OCl^-$ ratio at high pH. 2,4,6-Trichlorophenol was detected, but its level at high pH was only 33% that of the low pH sample. Larson and Rockwell (*40*) reported that two substituted benzoic acids common to natural waters, 4-hydroxybenzoic and vanillic acid, were decarboxylated by hypochlorite with the production of chlorophenols. 4-Hydroxybenzoic acid was rapidly converted to 2,4,6-trichlorophenol. Our laboratory tests have shown that phenol itself is not readily converted to chlorophenols by hypochlorous acid or hypochlorite, indicating that the detection of chlorophenols in the chlorinated humic acid extracts probably resulted from similar reactions with hydroxybenzoic acid moieties in the humic acid structure. Chloro- and bromothiopenes were also identified in these extracts, the mass spectra of which are included in Table III.

Table IV shows some preliminary data on the production of trihalomethanes, haloacetonitriles, and halopropanones resulting from the chlorination of Fluka humic acid in the presence of bromide at neutral pH. The results of GC/MS analyses of methylene chloride extracts show that the degree of bromine incorporation into trihalomethanes, haloacetonitriles, and halopropanones appears to be directly related to the amount of bromide present during chlorination. The results also indicate that the relative proportion of both haloacetonitriles and halopropanones to trihalomethanes decreases significantly with increasing bromide.

Summary and Conclusions

Figure 2 shows GC/MS chromatograms of a methylene chloride extract and of a closed-loop-stripping analysis of chlorinated Fluka humic acid (same batch solution) and compares them to a closed-loop-stripping analysis of a Cincinnati, OH, drinking water sample. The GC peak identifications and their mass spectra are shown in Table III. It is evident from the data presented in Tables II and III that the compounds formed as byproducts of chlorinations of humic material are similar to those found in actual drinking water samples. This similarity of compounds adds to the evidence that the chlorination of humic material in natural waters is responsible for the presence of a variety of halogenated organic compounds, including many with known mutagenic activity, which occur in drinking water as disinfection byproducts.

In conclusion, much research remains to be done. There is evidence that only a small fraction (about 6%) of the total mutagenic activity of a sample introduced into the gas chromatograph is represented by resolved GC peaks in the chromatogram. Many nonvolatile and/or labile compounds, which could be responsible for the majority of the mutagenic activity of chlorination byproducts, may go undetected when traditional gas-phase ionization methods are used. Extensive derivatization techniques to make the sample more gas chromatographable seems necessary. There is a need to develop and validate practical techniques for recovering highly polar compounds in an unaltered form and in a matrix suitable for subsequent chemical and biological characterization. Resin adsorption and liquid chromatography in conjunction with MS or MS/MS employing soft ionization techniques might be

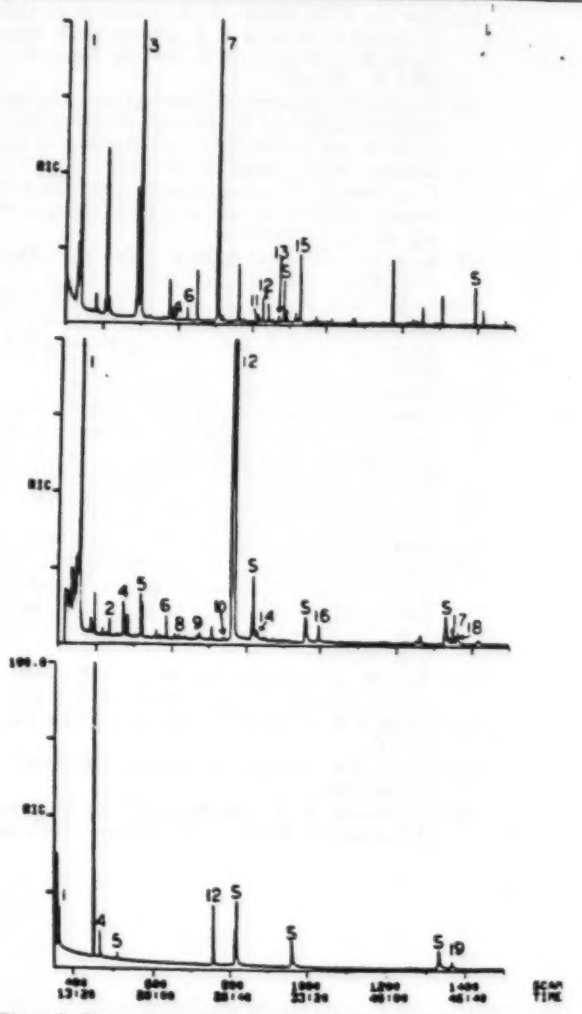

Figure 2. Reconstructed ion chromatograms of (upper) drinking water analyzed by closed-loop stripping, (middle) 8 mL of chlorinated Fluka humic acid solution diluted to 1 L with reagent water and analyzed by closed-loop stripping, and (lower) methylene chloride extract of a chlorinated Fluka humic acid solution (from same batch solution as middle sample). S = internal standards. Numbered GC peaks refer to identifications in Table III.

effective means of identifying and isolating more and larger quantities of biologically active compounds from aqueous solutions containing disinfectant byproducts.

Acknowledgments

We thank Melda Hirth and Lon Winchester for clerical assistance.

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Received August 15, 1983. Revised manuscript received March 29, 1984. Accepted April 16, 1984. Mention of trade names or commercial products is for identification only and does not imply endorsement by the U.S. Environmental Protection Agency.